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Use of Biological Assays in Short--Term Assessment of Inhaled Substances

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INTRODUCTION

Workers in the agricultural industry are exposed to an exceptionally wide variety of inhaled particles. These include fertilizers, pesticides, and herbicides as well as resuspended soil. Moreover, the composition of the soil (for example, the fraction which is free silica) varies from place to place. Other workers are exposed to complex grain dusts, such as that coming from various cereal grains (wheat, barley, rye, oats, corn), as well as various contaminants such as insects, mites, rodent debris, and fungi. This wide array of complex dusts presents problems in assessing the potential risk of various occupational exposures in agriculture.

In order to understand such exposures, it is possible to measure responses at the molecular, cell, organ, or organismic level. All approaches reflect the need to evaluate the toxicology of materials to which agricultural workers are exposed so that we can take appropriate preventive action. Government, unions, and industry now face the difficult task of assessing the toxicology of a wide variety of new chemicals and especially complex mixtures. The creativity of chemists who synthesize new compounds, the availability of new technologies, and finally the competitiveness of agriculture ensure that there will be a continuing stream of new aerosol exposures whose potential for damage must be assessed. Since they are new, epidemiology fails to provide information about health effects. Nevertheless, a guide to potential toxicity is needed to help design both appropriate control strategies and medical surveillance studies for humans employed in agriculture.

How can the risk of human pulmonary disease caused by exposure to complex and often poorly characterized dusts in the agricultural industry be predicted? Risk assessment may include: (1) air monitoring and physical and chemical characterization of collected dusts; (2) epidemiologic studies of humans; (3) controlled experimental exposures of humans in the laboratory; (4) chronic lifetime animal studies; (5) short-term animal bioassays; and (6) *in vitro* tests of mammalian cells. This paper emphasizes the fifth method of analysis and discusses the use of short-term animal bioassay systems to determine the health effects of inhaled particulates.

Animal studies have numerous advantages since ethical problems are minimized. The possibility of more serious disease can be assessed, and there are few limits to the invasiveness of the diagnostic procedures used. For example, long-term inhalation exposures of animals, followed by functional or histopathological studies of their lungs, have been used to study asbestos,[1] crystalline silica,[2] and coal dust.[3] A problem is that such studies are costly and time consuming. A typical lifetime study in rodents costs between 0.5 and 3 million dollars and may take 3 to 5 years to plan and complete. It is also difficult to obtain quantitative estimates of toxicity using standard pathological analyses. Morphometric measures based on extensive sampling of lung tissue as well as physiological or biochemical assessment may be required.

Clearly, there is a need for short-term tests. If large numbers of materials are to be analyzed, it is essential to have assays that are relatively inexpensive and that yield results in weeks or months, not years. Many investigators have promoted the use of *in vitro* assays to assess the potential toxicity of inhaled aerosols.[4-7] *In vitro* systems have advantages of reproducibility, cost, and specificity. Several tissue culture systems have been developed.[8,9] However, because the human pulmonary response to inhaled particles is the result of complex interactions involving many different cell types within the lung, the results obtained may be spurious. For example, inflammation involves recruitment of neutrophils, platelets, and serum proteins to the injured lung. Fibrogenesis involves the action of fibrogenesis–stimulating factors secreted by one cell (e.g., a macrophage) on another cell (a fibroblast). These essential interactions are rarely reproduced in any *in vitro* system.

Short-term *in vivo* assays can be considered as an alternative to short-term *in vitro* tests, because the short-term response of small animals to dusts is sufficiently similar to the human response to have predictive values when properly calibrated and interpreted. The major mechanisms of lung injury[10] are common to most mammals.

THE HAMSTER BIOASSAY

The hamster bioassay features the use of bronchoalveolar lavage (BAL). During the last decade, BAL has been used increasingly to assess lung injury in animals and man. BAL has been employed to discriminate among toxic agents such as metal salts or mineral dusts.[11,12] Key issues in the application of BAL to inhalation toxicology are the specificity and sensitivity of the procedure. What is the smallest amount of dust which causes a measurable response? More important, what is the ability of BAL to discriminate among dusts of varying toxicities and those producing different resulting lesions? To what extent does BAL have predictive value? Can one examine acute events and describe long-term irreversible chronic changes?

We have developed a short–term (1 to 30 d postexposure) animal bioassay system in which the toxicity of a particular dust may be estimated by comparison to known dusts with a

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demonstrated range of toxicities for human pulmonary disease. We employ hamsters exposed to dusts by either inhalation or by intratracheal instillation[13] and quantify the response by measuring biochemical and cellular indicators in BAL fluid. The parameters measured represent a wide spectrum of possible responses to inhaled particles, including inflammation, pulmonary edema, cellular damage, cellular secretion, and endocytic capacity of pulmonary macrophages. We have calibrated the system with dusts for which there is considerable human experience. Cellular and biochemical changes were measured in BAL of hamsters after exposure to α-quartz, iron oxide, and aluminum oxide.[12] α–Quartz is a highly toxic, fibrogenic mineral dust, whereas aluminum oxide and iron oxide are both of low toxicity.

One day after exposure, the levels of β–*N*–acetylglucosaminidase were significantly elevated by exposure to the 0.75– and 3.75–mg doses of all three dusts (see Figure 1). However, the response to α–quartz was greater than the response to the other two dusts, especially at the highest dose. β–*N*–acetylglocosaminidase is an example of a lysosomal enzyme that is released from cells during phagocytosis, cell injury, or cell death.[14] Polymorphonuclear neutrophils (PMNs),[14] macrophages, and type II cells[15] all contain acid hydrolases. Excessive release of lysosomal enzymes may elicit unwanted proteolysis from cathepsins or membrane destruction by phospholipases.

α–Quartz also elevated albumin levels in lavage fluid at both 0.75– and 3.75–mg doses as shown in Figure 2. The highest dose caused a more than 40-fold increase above control levels. Aluminum oxide and iron oxide were also associated with an increase at 3.75 mg, but albumin levels clearly distinguished between these relatively nontoxic dusts and the highly fibrogenic α–quartz. Albumin is primarily a

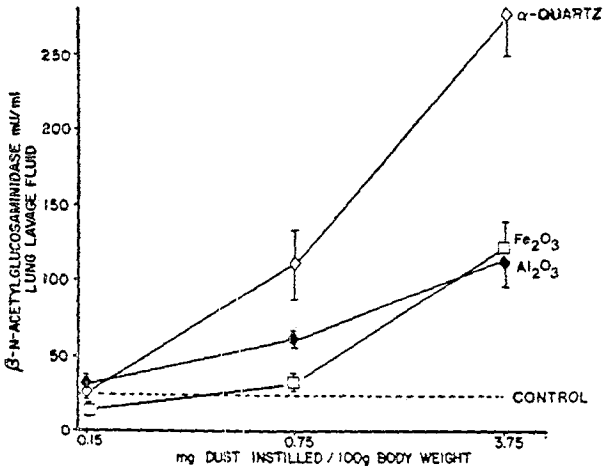

FIGURE 1. Dose-response curve for β–*N*-acetylglucosaminidase 1 d after instillation of particles. $p < 0.01$ for all points except 0.75 mg iron oxide and 0.15 mg aluminum oxide ($p < 0.05$), and 0.15 mg α–quartz (not significant). Values are mean ± standard errors. (Adapted from Beck, B. D., Brain, J. D., and Bohannon, D. E., *Exp. Lung Res.*, 2, 289, 1981. With permission.)

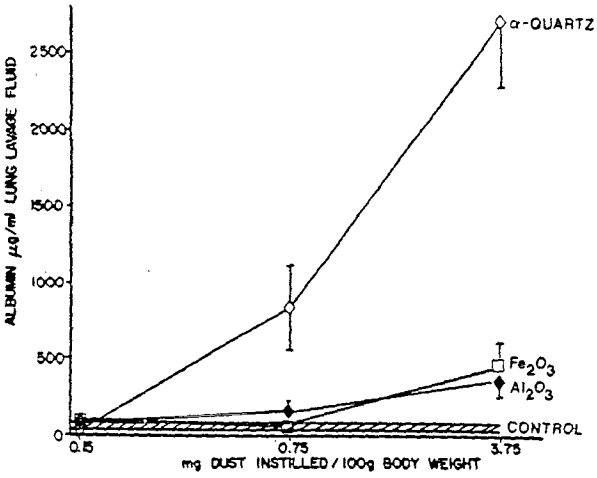

FIGURE 2. Dose-response curve for albumin in extracellular supernatant of lung lavage fluid 1 d after exposure to iron oxide, aluminum oxide, or α–quartz. The Wilcoxon rank-sum test was used to compare experimentals and saline–only controls. $p < 0.01$ for all points except 0.75 mg aluminum oxide and all 0.15–mg samples (not significant). Values represent mean ± standard errors. (Adapted from Beck, B. D., Brain, J. D., and Bohannon, D. E., *Exp. Lung. Res.*, 2, 289, 1981. With permission.)

serum protein whose presence in BAL is due to passage across damaged endothelial and epithelial barriers. Albumin is usually the most abundant protein in BAL.[16,17] Elevated albumin levels indicate pulmonary edema, a common manifestation of acute pulmonary injury.[12,18]

Figure 3 illutratres that α–quartz also causes depressed macrophage function. The lambda values shown are the fraction of radioactive gold colloid which was ingested 90 min after it had been instilled through the trachea. Brain and Corkery[19] provide details of this assay which estimate the endocytic activity of macrophages *in situ*. At a dose of 3.75 mg of α–quartz, less than 30% of the gold was ingested; iron oxide and aluminum oxide have no significant effect on lambda.

The full bioassay includes a number of other parameters such as peroxidase, elastase; hemoglobin, as well as the numbers of erythrocytes, neutrophils, and macrophages. An essential aspect of bioassays like this is to compare the responses of unknown dusts with other well–characterized standards. Both positive and negative controls should be used. The best calibrating materials would be those for which there is a considerable experience in humans such as the dusts shown in Figures 1 to 3. Then the type and intensity of response for a new unknown dust could be compared to these standards.

A key feature of assays utilizing lung lavage is the time course of the response. Some agents will yield similar responses when examined soon after exposure. However, the more toxic material may frequently exhibit a more persistent change in the cellular and enzymatic parameters than nontoxic controls. For example, there was a prolonged elevation in the numbers of macrophages and PMNs with quartz, but not with iron oxide. PMN numbers in the lung lavage fluid were

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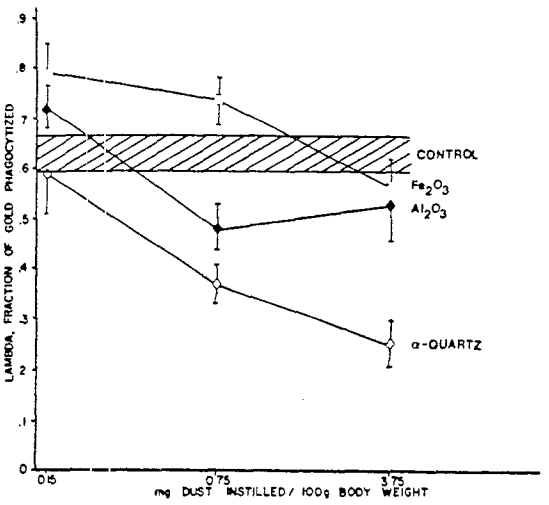

FIGURE 3. Dose-response curve for lambda assay 1 d after exposure to iron oxide, aluminum oxide, or α–quartz. *The Wilcoxon* rank-sum test was used to compare experimentals and saline only controls. $p < 0.01$ for 0.75 and 3.75 mg α–quartz, 0.75 mg aluminum oxide; $p < 0.05$ for 0.15 mg iron oxide. Values represent mean ± standard errors. (Adapted from Beck, B. D., Brain, J. D., and Bohannon, D. E., *Exp. Lung. Res.*, 2, 289, 1981. With permission.)

highest 4 d after exposure to α–quartz, although after 2 weeks they still had not approached control levels.[12]

A somewhat different pattern was observed for lactate dehydrogenase (LDH) in lavage fluid. This is a cytoplasmic enzyme involved in energy metabolism; its extracellular release is associated with cell injury or death. LDH levels in lung lavage fluid were highest 1 d after exposure to both iron oxide and α–quartz. In time, LDH levels declined signficantly in the quartz-exposed animals and only slightly in the iron oxide-exposed animals. Nevertheless, the levels in the quartz animals remained higher than those in the iron oxide-exposed animals at all times. These effects were observed at relatively low levels of quartz compared to levels used in animal models of chronic silicosis.

Application of this system to dusts produced by the eruption of Mt. St. Helens volcanic ash suggested that volcanic ash has low to moderate toxicity.[20] We concluded that adverse health effects in human populations are unlikely except with high or prolonged exposure. Surfactant levels in BAL in rats after quartz and Mt. St. Helens volcanic ash exposure have been studied by Martin and co–workers.[21] Quartz causes a prolonged elevation in PMN numbers and surfactant levels. The effects were much less marked with volcanic ash than with quartz. These observations are consistent with histopathological studies of lungs of exposed animals which demonstrated much greater fibrogenicity of α–quartz than of volcanic ash. These studies show the usefulness of BAL in providing a rapid evaluation of the toxicity of poorly characterized samples. Useful results can be obtained even when chemical analyses of epidemiological studies are not available for toxicity estimates.

IDENTIFYING SOURCES OF DAMAGE INDICATORS: LDH ISOENZYMES

We are searching for other ways of making the assay more interpretable. As discussed earlier, LDH is released from cells in response to toxic particles. However, if LDH is recovered in the cell–free supernatant of lung lavage fluid, where does it come from? Is the source inflammatory cells (macrophages or PMNs), serum, epithelial cells, or endothelial cells? Beck et al.[22] have used isoenzyme analysis to infer the sources of LDH.

To differentiate among types of injury, we monitored changes in LDH isoenzyme patterns in BAL after a range of injuries: α–quartz, hyperoxia, the detergent Triton X-100, and SO_2. The LDH isoenzyme patterns in BAL were evaluated and compared with patterns from hamster lung homogenates, red blood cells, macrophages, PMNs, type II cells, and serum. The isoenzyme pattern in BAL from quartz–exposed animals resembled that of the PMNs and macrophages, suggesting phagocytic cell death. In contrast, BAL from Triton X-100–treated animals had an isoenzyme pattern similar to that of the lung homogenate and red blood cells. Exposure to 100% O_2 for 4 d produced an isoenzyme pattern similar to serum, an observation consistent with the demonstrated effects of O_2 on the capillary endothelium.

Figure 4 presents graphically the percentage of each LD isoenzyme from serum or from lung lavage fluid of Syrian golden hamsters exposed to 100% O_2 for 96 h. The distribution of the five LD isoenzymes is similar and consistent with the hypothesis that oxygen toxicity caused damage to the air-blood barrier. Serum LD and other serum proteins leaked into alveolar spaces and were subsequently recovered by lavage.

In Figure 5 the LD pattern is shown for: (1) supernatant from BAL recovered from hamsters exposed to iron oxide aerosol and (2) hamster peritoneal PMNs. The LD patterns shown in Figure 5 are markedly different from those seen in Figure 4. For example, there is little LD1 (<3%), but a great deal of LD5 (~60%). The similarity in pattern suggests that the

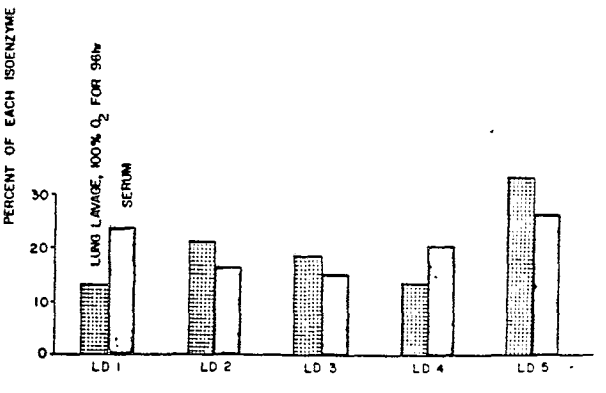

FIGURE 4. Comparison of LD isoenzyme patterns from hamster serum and from lung lavage fluid of hamsters exposed to 100% O_2 for 96 h. (Adapted from Beck, B. D., Gerson, B., Feldman, H. A., and Brain, J. D., *Toxicol. Appl. Pharmacol.*, 71, 59, 1983. With permission.)

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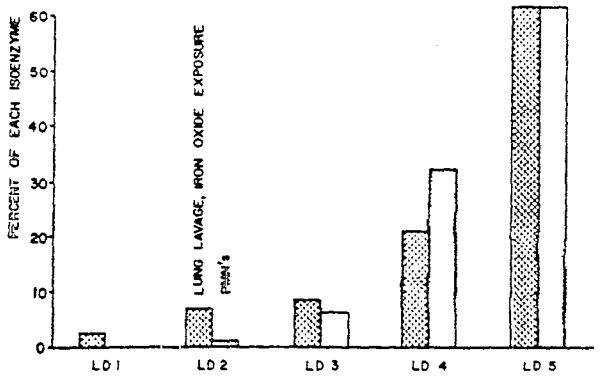

FIGURE 5. Comparison of LD isoenzyme patterns from hamster peritoneal PMNs and from lung lavage fluid of hamsters exposed to 3.75 mg iron oxide per 100 g body weight. (Adapted from Beck, B. D., Gerson, B., Feldman, H. A., and Brain, J. D., *Toxicol. Appl. Pharmacol.*, 71, 59, 1983. With permission.)

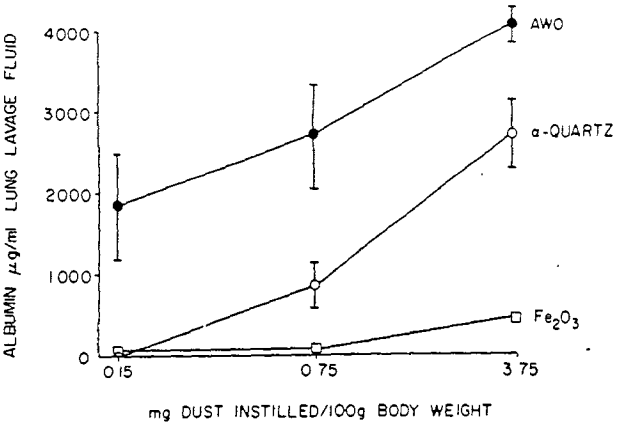

FIGURE 6. Concentration of albumin in the cell-free supernatant of BAL fluid. The effects of iron oxide, α–quartz, and combustion products of AWO are shown 1 d after intratracheal instillation. Values are mean ± standard errors. (Adapted from Beck, B. D., Brain, J. D., and Wolfthal, S. F., *Inhaled Particles IV*, Dodgson, J., Ed., British Occupational Hygiene Society, Edinburgh, Scotland.)

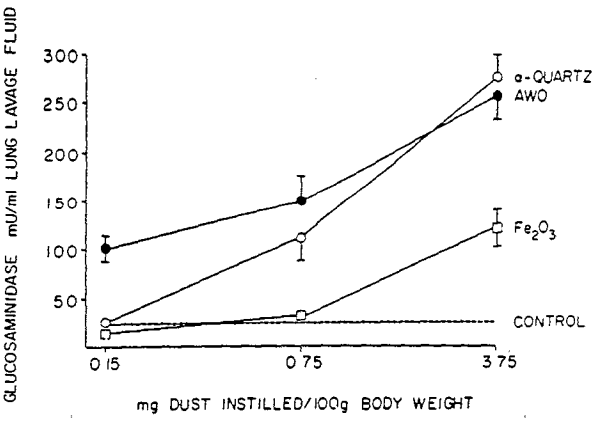

FIGURE 7. Concentration of β–*N*–glucosaminidase in the cell-free supernatant of lavage fluid after exposure to iron oxide, α–quartz, and AWO. Values are mean ± standard errors. (Adapted from Beck, B. D., Brain, J. D., and Wolfthal, S. F., *Inhaled Particles IV*, Dodgson, J., Ed., British Occupational Hygiene Society, Edinburgh, Scotland.)

LD could be coming from PMNs. Macrophages have a similar LD composition, so they also may be a source.

AUTOMOBILE WASTE OIL COMBUSTION PRODUCTS

This assay is particularly suited for analyzing new complex agents which are just being introduced into the environment. We have recently investigated the pulmonary toxicity of respirable particulates from an air–atomizing oil space heater using automobile waste crankcase oil (AWO).[23] A combustion sample was prepared from AWO from a service station by Dr. R. E. Hall of the U. S. Environmental Protection Agency, using an air–atomizing oil burner rated at 250,000 BTU/h heat input. Respirable particulates were collected from a dilution tunnel by electrostatic precipitation using a massive air volume sampler.[24] Analysis of the particles showed certain metals were present at relatively high levels, for example: Pb, 75.6 mg/g; Zn, 23.0 mg/g; and Fe, 5.3 mg/g.

At 1 d postexposure, there was extensive pulmonary injury as demonstrated by cellular and biochemical indicators in BAL: (1) elevated levels of albumin, (2) increased extracellular glucosaminidase, and (3) impaired pulmonary macrophage phagocytosis. The injury was often greater than that seen in response to toxic α–quartz. Some of the data obtained are shown in Figures 6 to 8.

However, assays of BAL up to 14 d post-AWO exposure demonstrated that most indicators rapidly approached control values. This is in contrast to the persistent inflammation caused by α–quartz. As shown in Figure 9, LDH values approached control values at 2 weeks after intratracheal instillation of AWO. Following quartz exposure, the LDH level remains elevated. This suggests that the toxic effects of AWO stem from soluble components which are rapidly cleared. AWO may be less likely to cause chronic pulmonary disease than α–quartz unless exposure persists. Acute injury as manifested by bronchitis or increased susceptibility to infection may be a more likely outcome than fibrosis.

By comparing the response to AWO with the response to the same doses of toxic α–quartz and nontoxic iron oxide, we conclude that the AWO combustion products have a high potential to cause acute lung injury. Both soluble and insoluable components of AWO can produce lung injury. Some, but not all, of these effects are due to acidity and divalent cations, such as lead, which are present at high levels.

CONCLUSION

Experimental pathology has frequently advanced because of the addition of new diagnostic tools. During the last decade, BAL has emerged as a very useful tool in the assessment of lung injury. It is applicable to both animal models exposed to inhaled particles and gases in a laboratory and to humans

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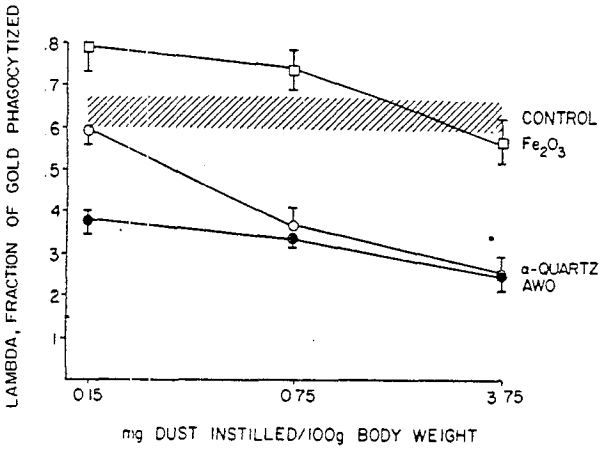

FIGURE 8. The fraction of gold particles, lambda ingested by macrophages *in situ*, is shown. Measurements were made 1 d after exposure to iron oxide, α–quartz, and AWO. Values are mean ± standard errors. (Adapted from Beck, B. D., Brain, J. D., and Wolfthal, S. F., *Inhaled Particles IV*, Dodgson, J., Ed., British Occupational Hygiene Society, Edinburgh, Scotland.)

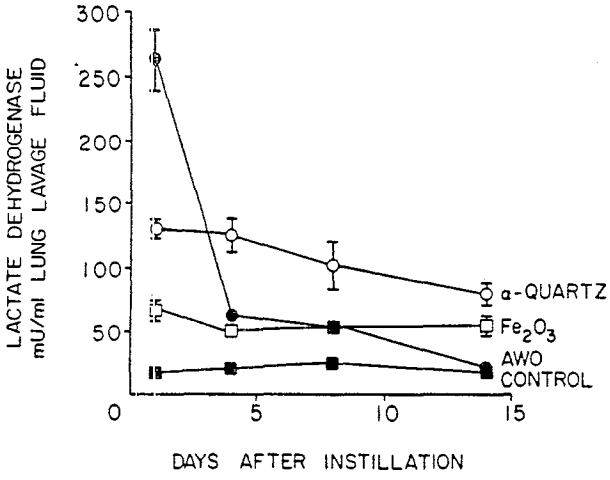

FIGURE 9. Time course for LDH in the extracellular supernatant fraction of lung lavage fluid after exposure to 3.75 mg iron oxide, α–quartz, or AWO per 100 g body weight. Values are mean ± standard errors. (Adapted from Beck, B. D., Brain, J. D., and Wolfthal, S. F., *Inhaled Particles IV*, Dodgson, J., Ed., British Occupational Hygiene Society, Edinburgh, Scotland.)

encountering exposures to the same agents in occupational and urban environments. Information can be gathered from BAL relating to the extent and type of lung injury and the mechanisms involved. Needed are more extensive comparisons of injury as judged by other approaches with the results of BAL. For example, short-term bioassay results can be integrated with industrial hygiene and epidemiology results as was done in a recent study of talc and granite dusts.[25] It is also likely that other constituents of BAL can be quantified which will help make bioassays utilizing BAL more specific and sensitive. The use of BAL in short-term animal assays can be an important source of information regarding the toxicity of new and poorly characterized inhaled particles.

REFERENCES

1. **Brody, A.R. and DeNee, P.B.,** Biological activity of inorganic particles in the lung, *CRC Crit. Rev. Toxicol.*, 7, 277, 1981.
2. **Gross, P., DeVilliers, A.J., and deTrevelle, R.T.P.,** Experimental silicosis, *Arch. Pathol.*, 84, 87, 1967.
3. **Busch, R.H., Filipy, R.E., Karagianes, M.T., and Palmer, R.F.,** Pathologic changes associated with experimenal exposure of rats to coal dust, *Environ. Res.*, 24, 53, 1981.
4. **Dean, J.H., Boorman, G.A., Luster, M.I., Adkins, B., Jr., Lauer, L.D., and Adams, D.O.,** Effect of agents of environmental concern on macrophage functions, in *Mononuclear Phagocyte Biology*, Volkman, A., Ed., Marcel Dekker, New York, 1984, 473.
5. **Liu, W.K., Tsao, S.W., and Wong, J.W.C.,** In vitro effects of fly ash on alveolar macrophages, *Conservation Recycling*, 7, 361, 1984.
6. **Snella, M.-C.,** Manganese dioxide induces alveolar macrophage chemotaxis for neturophils in vitro, *Toxicology*, 34, 153, 1985.
7. **Hatch, G.E., Boykin, E., Graham, J.A., Kewtas, J., Pott, F., Loud, K., and Mumford, J.L.,** Inhalable particles and pulmonary host defense: in vivo and in vitro effects of ambient air and combustion particles, *Environ. Res.*, 36, 67, 1985.
8. **Kaw, J.L.,** Tissue culture in pneumoconiosis, *CRC Crit. Rev. Toxicol.*, 5, 103, 1977.
9. **Miller, K.,** The effects of asbestos on macrophages, *CRC Crit. Rev. Toxicol.*, 5, 319, 1978.
10. **Fantone, J.C. and Ward, P.A.,** Mechanisms of lung parenchymal injury, *Am. Rev. Respir. Dis.*, 130, 484, 1984.
11. **Henderson, R.F., Rebar, A.H., Pickrell, J.A., and Neulton, G.J.,** Early damage indicators in the lung. III. Biochemical and cytological response of the lung to inhaled metal salts, *Toxicol. Appl. Pharmacol.*, 51, 123, 1979.
12. **Beck, B.D., Brain, J.D., and Bohannon, D.E.,** An in vivo hamster bioassay to assess the toxicity of particulates for the lungs, *Toxicol. Appl. Pharmacol.*, 66, 9, 1982.
13. **Brain, J.D., Knudson, D.E., Sorokin, S.P., and Davis, M.A.,** Pulmonary distribution of particles given by intratracheal instillation or by aerosol inhalation, *Environ. Res.*, 11, 13, 1976.
14. **Weissman, G., Smolin, J.E., and Korchak, H.M.,** Release of inflammatory mediators from stimulated neutrophils, *N. Engl. J. Med.*, 303, 27, 1980.
15. **Hook, G.E.R.,** Extracellular hydrolases of the lung, *Biochemistry*, 17, 520, 1978.
16. **Bell, D.Y., Haseman, J.A., Spock, A., McLennan, G., and Hook, G.E.R.,** Plasma proteins of the bronchoalveolar surface of the lungs of smokers and nonsmokers, *Am. Rev. Respir. Dis.*, 124, 72, 1981.
17. **Merrill, W., O'Hearn, E., Rankin, J., Naegel, G., Matthay, R.A., and Reynolds, H.Y.,** Kinetic analysis of respiratory tract proteins recovered during a sequential lavage protocol, *Am. Rev. Respir. Dis.*, 126, 617, 1982.
18. **Chichester, C.O., Palmer, K.C., Hayes, J.A., and Kagen, H.M.,** Lung lysyl oxidase and prolyl hydroxylase: increases induced by cadmium chloride inhalation and the effect of beta-aminopropionitrile in rats, *Am. Rev. Respir. Dis.*, 124, 709, 1981.
19. **Brain, J.D. and Corkery, G.C.,** The effect of increased particles on the endocytosis of radiocolloids by pulmonary macrophages *in vitro*: competitive and cytotoxic effects, in *Inhaled Particles IV*, Walton, W.H., Ed., Perfamon, New York, 1977, 551.
20. **Beck, B.D., Brain, J.D., and Bohannon, D.E.,** The pulmonary toxicity of an ash sample from Mt. St. Helens volcano, *Exp. Lung Res.*, 2, 289, 1981.
21. **Martin, T.R., Chi, E.Y., Covert, D.S., Hodson, W.A., Kessler, D.E., Moore, W.E., Altman, L.C., and Butler, J.,** Comparative effects of inhaled volcanic ash and quartz in rats, *Am. Rev. Respir.*

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Dis., 128, 144, 1983.

22. **Beck, B.D., Gerson, B., Feldman, H.A., and Brain, J.D.,** Lactic dehydrogenase isoenzymes in hamster lung lavage fluid after lung injury, *Toxicol. Appl. Pharmacol.*, 71, 59, 1983.
23. **Beck, B.D., Brain, J.D., and Wolfthal, S.F.,** Assessment of lung injury produced by particulate emissions of space heaters burning automobile waste oil, in *Inhaled Particles VI*, Dodgson, J., Ed., British Occupational Hygiene Society, Edinburgh, Scotland, in press.
24. **Hall, R.E., Crooke, M.W., and Barbour, R.L.,** Comparison of air pollutant emissions from vaporizing and air atomizing waste oil heaters, *J. Air Pollut. Control Assoc.*, 33, 683, 1983.
25. **Beck, B.D., Feldman, H.A., Brain, J.D., Smith, T.J., Hallock, M., and Gerson, B.,** The pulmonary toxicity of talc and granite dust as estimated from an *in vivo* hamster bioassay, *Toxicol. Appl. Pharmacol.*, 87, 222, 1987.

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